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Quantity of Cytomegalovirus Viruria Is a Major Risk Factor for Cytomegalovirus Disease After Renal Transplantation

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Studies have shown that risk factors for human cytomegalovirus (HCMV) disease after renal transplant include primary infection (virus of donor origin infecting a non-immune individual), re-infection (virus of donor origin infecting a immune individual), and the detection of viraemia (as a marker of virus dissemination). We now report that viral load in the urine is also a significant factor in HCMV disease and is one of the main mechanisms underlying the risk associated with viraemia and donor serostatus. Longitudinal analysis of a group of 196 renal recipient identified 35 recipients who were PCR positive for HCMV in urine. Elevated viral loads were present in symptomatic patients, viraemic patients, and patients experiencing primary HCMV infection. Disease was associated with the peak quantity of virus present in the urine during the post-transplant period ($P = 0.0001$), with viraemia ($P = 0.0003$), and with transplantation of a seropositive donor ($P = 0.03$). Univariate logistic regression analysis showed that increases of 0.25 $\log_{10}$ in viral load were associated with a 179% increased risk of disease (odds ratio = 2.79; 95% C.I. 1.22-6.39; $P = 0.02$). This effect persisted in a multivariate logistic analysis when viraemia was incorporated (odds ratio = 2.77; 95% C.I. 1.07-7.18; $P = 0.04$). In contrast, the significant association between viraemia and disease observed in univariate analysis (odds ratio = 23.75; 95% C.I. 3.69-152.90; $P = 0.0009$) became marginally non-significant in multivariate analysis once viral load had been controlled for (odds ratio = 34.54; 95% C.I. 0.75-1599.00; $P = 0.07$). The computed probability of disease showed that a rapid transition occurred at viral loads between $10^{5.7}$ and $10^{6.5}$ genomes/ml urine in non-viraemic patients compared to viral loads between $10^{5.0}$ and $10^{5.7}$ genomes/ml urine in patients with concurrent viraemia. The implications of these findings for understanding HCMV pathogenesis, improving patient management, and optimising trials of antiviral treatment are discussed. ***J. Med. Virol. 52:200–205, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: quantitation; QCPCR; glycoprotein b

INTRODUCTION

Approximately 60% of adults in developed countries and 100% of adults in developing countries have been infected with human cytomegalovirus (HCMV). In virtually all of these cases the primary infection is silent clinically. However, if a patient is immunocompromised due to immunological immaturity (fetus), HIV infection, or receipt of immunosuppressive drugs to permit organ transplantation, HCMV infection is common and represents a major cause of morbidity and mortality in each patient group [Griffiths, 1995]. For example, between 15% and 50% of patients excrete HCMV after renal transplant, 30% to 40% of whom will develop HCMV disease [Balfour et al., 1989; Betts et al., 1977]. Sources of HCMV infection include the kidney donor or the recipient. Studies have shown consistently that primary infection acquired from the donor is associated with HCMV disease, whereas reactivation of latent recipient virus has a significantly lower risk [Betts et al., 1977; Grundy et al., 1988; Rangan et al. 1991]. By restriction-enzyme typing strains of HCMV excreted by pairs of recipients receiving kidneys from

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the same donor, it has been shown that re-infection (donor virus infecting an immune individual) is associated with a risk of disease intermediate between primary infection and reactivation [Grundy et al., 1988]. Several studies have also shown that detection of HCMV viraemia is a significant risk factor for HCMV disease [Betts et al., 1977; Cheeseman et al., 1979; Pillay et al., 1993].

With respect to quantitative measures of HCMV, 21 years ago, Stagno et al. [1975] showed that the titre of HCMV in the urine was significantly associated with disease following congenital HCMV infection. More recently, data generated using molecular methods have indicated the importance of HCMV load for disease in recipients of solid organs and HIV-infected individuals [Fox et al., 1995; Kuhn et al., 1994; Saltzman et al., 1992; Schafer et al., 1993]. Following intensive prospective longitudinal monitoring of our renal transplant recipients using qualitative and quantitative polymerase chain reaction (PCR) methods, we now report that viral load is one of the major mechanisms underlying HCMV disease and provides potentially a marker to assess the probability of future disease.

MATERIALS AND METHODS

Study Population

Between July 1992 and December 1994, a group of 196 renal transplant patients were followed prospectively for evidence of HCMV infection by PCR as described previously [Kidd et al., 1993]. Urine and blood were collected weekly from in-patients, and at every subsequent out-patient visit. The total number of urine samples from these patients analysed for HCMV by PCR was 2,968 (median per patient = 7; range, 1–62). Urine was analysed without processing, whereas HCMV in the blood was extracted using a commercial DNA extraction procedure (Qiagen, Germany) and detected subsequently by PCR. The term *viraemia* is used here to describe PCR detection of HCMV in blood samples.

HCMV was detected by PCR in the urine of 35 transplant recipients, indicating HCMV replication, and these patients were selected for detailed study of virus load using a quantitative-competitive PCR on unprocessed urine [described in detail in Fox et al., 1992]. Twenty-seven patients had 3 or more consecutive PCR positive samples; 8 had urine samples which were positive for HCMV DNA in a non-consecutive sequence, two of which were symptomatic; and 161 patients were PCR-negative and asymptomatic. In each of the 35 positive patients, viral load was plotted as a function of time and the peak level of genomes/ml of urine during the post-transplant surveillance period was recorded. The longitudinal fluctuations in viral load for 12 of these patients have been described previously [Fox et al., 1995]. The median age of the 35 patients was 14 years (range, 2–57). There was no difference in disease incidence in paediatric versus adult recipients (P = 0.3, Fisher's exact test) or between patients above or below the median age (P = 0.35). The median number of urine samples analysed by quantitative PCR from these 35 patients was 12 (range, 4–39) and was comparable between the asymptomatic patients (n = 23; median sample per patient = 10; range, 4–36) and symptomatic patients (n = 12; median samples per patient = 14; range, 4–39). All 35 patients were receiving their first renal transplant.

The immunosuppressive regimen used in the adult and paediatric renal transplant recipients including the management of rejection episodes has been described previously [Irragorri et al., 1993; Pillay et al., 1993]. Briefly, paediatric transplant recipients received prednisolone (60 mg/m^2/day i.v.) preoperatively, reducing to 10 mg/m^2/day by 4–6 weeks, and the same dose but on alternate days by 8 weeks post-transplant. Patients also received azathioprine (60 mg/m^2) 24 hours post-transplant and cyclosporin A (50 mg/m^2 i.v.) pre-operatively then post-operatively to maintain plasma levels at 50–100 ng/ml. Rejection in the paediatric patients was treated with methylprednisolone (600 mg/m^2/day) for 3 days during the first 6 weeks post-transplant and high-dose oral prednisolone (3 mg/kg/day) for 3 days thereafter. Steroid-resistant rejection was treated with ATG (2 mg/kg/day) for 10 days. Adult transplant recipients received daily immunosuppressive therapy of prednisolone (0.3 m g/kg), ATG (1 mg/kg), and cyclosporin A (10–15 mg/kg). First rejection episodes were treated with methylprednisolone (500 mg/kg/day i.v.) for 3 days and second episodes with 1 g of the same, administered on 3 consecutive days, and further episodes with ATG (5 mg/kg) for 10–15 days. Twenty-three adult recipients who received a chimeric anti-CD25 antibody in addition to their baseline immunosuppression were included in the present study [Amlot et al., 1995].

HCMV Disease and Antiviral Chemotherapy

HCMV disease was diagnosed when typical features occurred within 2 weeks of the detection of HCMV infection as follows:

Debilitating febrile illness. Fever spiking to 38°C or above for at least 48 hours in the absence of rejection or bacterial/fungal infection, with neutropenia, in association with HCMV viraemia.

Hepatitis. Alteration in liver function tests (AST > twice upper limit of normal) on at least 2 consecutive days in absence of bacterial or fungal infection, in association with HCMV shedding from any site.

Pneumonitis. Chest symptoms and/or a characteristic chest X-ray pattern unresponsive to antibiotics and with evidence of HCMV infection in bronchoalveolar lavage fluid.

The period between transplant and onset of disease in the 12 symptomatic patients was as follows: 11, 12, 21, 24, 32, 33, 36, 36, 75, 99, 120, and 370 days.

Six of the 35 patients analysed in detail were prescribed antiviral chemotherapy comprising ganciclovir (3 patients) and acyclovir (5 patients) according to established dosage regimens. Antiviral therapy was initiated at the onset of symptoms rather than pre-

TABLE I. Maximum Median Viral Load, Disease Status, and Viraemia Status of the 35 Patients Who Exhibited HCMV in Their Urine Post-Transplant*

Sero-status	Disease	Viral load ($\log_{10}$ genomes/ml urine)
D+R−	Yes	7.1*,6.9*,6.85*,6.55*,6.2*,5.9,5.85*,5.8*,
	No	6.55,5.75,5.5,5.3,5.25*,4.0*,5.3
D+R+	Yes	6.85*,6.6*,6.3*.6.33*
	No	5.55,3.95,3.8,3.75,3.55*,3.7,4.14,3.1
D−R+	Yes	—
	No	4.85,4.55,4.25,3.6,3.7*,3.7,3.1,3.1

*Patients with viraemia.

emptively. In addition, 3 patients received immunoglobulin for HCMV pneumonitis. None of the remaining 161 patients included in this study received antiviral therapy.

Statistical Methods

The significance of differences between maximum viral loads in symptomatic and asymptomatic patients, D+R−, D+R+, and D−R+ groups or patients with or without viraemia was assessed by the Mann-Whitney test [Altman, 1993]. Initially, univariate relationships were studied by drawing up two-by-two contingency tables of HCMV disease (yes/no) against either viraemia (yes/no) or donor serostatus (positive/negative). The relationships between these variables were then tested for significance using chi-squared test (viraemia) or Fischer's exact test (donor status). Viral load differences between these with and without HCMV disease were studied using the Mann-Whitney U test. Where significant, these relationships were then quantified using univariate and multivariate logistic regression analysis [Altman, 1993]. For these analyses; the odds ratios quoted refer to a 0.25 $\log_{10}$ increase in viral load. The results were used to generate an equation to determine the expected probability of being symptomatic at any given viral load, and predicted probabilities from the final logistic models were plotted against viral load. All analyses were carried out using PRO LOGIST in the statistical analysis system [SAS, 1989].

RESULTS

Of the 196 renal transplant recipients assessed, 35 patients were HCMV PCR positive in urine during post-transplant surveillance; 12 of these patients exhibited HCMV disease. The maximal virus load present in the urine of the 35 patients during the post-transplantation surveillance period ranged from $10^{3.1}$ to $10^{7.1}$ genomes/ml (median $10^{5.2}$ genomes/ml) and are detailed in Table I, together with the donor/recipient serostatus and whether viraemia was present.

The relationship between maximum HCMV load in the urine post-transplantation with HCMV-related disease, viraemia, and donor/recipient serostatus for HCMV is shown in Figure 1. The median peak viral load in the urine of symptomatic patients was $10^{6.44}$ genomes/ml (range $10^{5.8}$–$10^{7.1}$ genomes/ml), which was 275-fold higher than the median peak viral load in the urine of asymptomatic patients ($10^{4.0}$ genomes/ml: range, $10^{3.1}$–$10^{6.55}$ genomes/ml; $P < 0.01$, Mann-Whitney test; Fig. 1A). Viraemic patients had a 10-fold higher viral load (median, $10^{6.25}$ genomes/ml) in their urine compared to patients without viraemia (median load, $10^{5.25}$ genomes/ml; $P = 0.004$, Mann-Whitney test, Fig. 1B). Maximal viral load was also significantly higher in patients with primary infection (D+R− group, n = 15; median load, $10^{5.85}$ genomes/ml) compared to those with reactivation of latent infection (D−R+ group, n = 8; median load, $10^{3.725}$ genomes/ml; $P < 0.01$, Mann-Whitney test), corresponding to a 133-fold difference (Fig. 1C), and in patients at risk of both primary infection or reinfection (D+R+ group, n = 12; median load, $10^{4.025}$ genomes/ml; $P < 0.05$). Interestingly, in the latter group, 2 populations of viral load were apparent; the first population, none of whom had disease, had a level of HCMV in the urine comparable to the median levels detected in the D−R+ group, i.e., reactivation of recipient virus (median load, $10^{3.8}$ genomes/ml). The other population exhibited a significantly higher HCMV load ($P < 0.01$, Mann-Whitney test; median, $10^{6.33}$ genomes/ml) comparable to the median level detected in the D+R− group indicative of reinfection by donor virus strains.

Univariate analysis showed that viral load, viraemia, and receipt of an organ from a HCMV seropositive donor were all associated with an increased risk of disease ($P = 0.0001$, $P = 0.0003$, and $P = 0.03$, respectively), while reactivation of recipient virus was associated with a lower risk of disease ($P = 0.04$). The odds ratios associated with increases in viral load, viraemia, and recipient seropositivity were calculated using both univariate and multivariate logistic regression models and are shown in Table II. In the univariate analysis, each 0.25 $\log_{10}$ increase in viral load was associated with a 179% increase in the risk of HCMV disease ($P = 0.02$), and viraemia was associated with an odds ratio of 23.75 ($P = 0.0009$) and recipient status with an odds ratio of 0.22 ($p = 0.05$). In the multivariate analysis, adjusting for viraemia affected the odds ratio associated with viral load only marginally (OR 2.77; $P = 0.04$), whereas controlling for viral load markedly reduced the significance of viraemia for HCMV disease ($P = 0.07$), despite increasing the odds ratio from 23.75 to 34.54. The significant protective effect of recipient seropositivity for disease was negated in the multivariate analysis after controlling for either viral load or viraemia.

The expected probability of suffering HCMV disease at any given viral load for all 35 patients is shown in Figure 2A. This graph illustrates that a sharp transition in risk occurs between $10^{5.5}$ and 10^{6} genomes/ml urine, resulting in a large increase in the probability of disease for relatively modest increases in viral load (viral load at 50%, $P = 10^{5.8}$ genomes/ml urine). When a similar graph was produced based on the multivariate analysis (Fig. 2B), the viral load yielding a 50% probability of disease for viraemic and non-viraemic patients was separated by 0.8 $\log_{10}$.

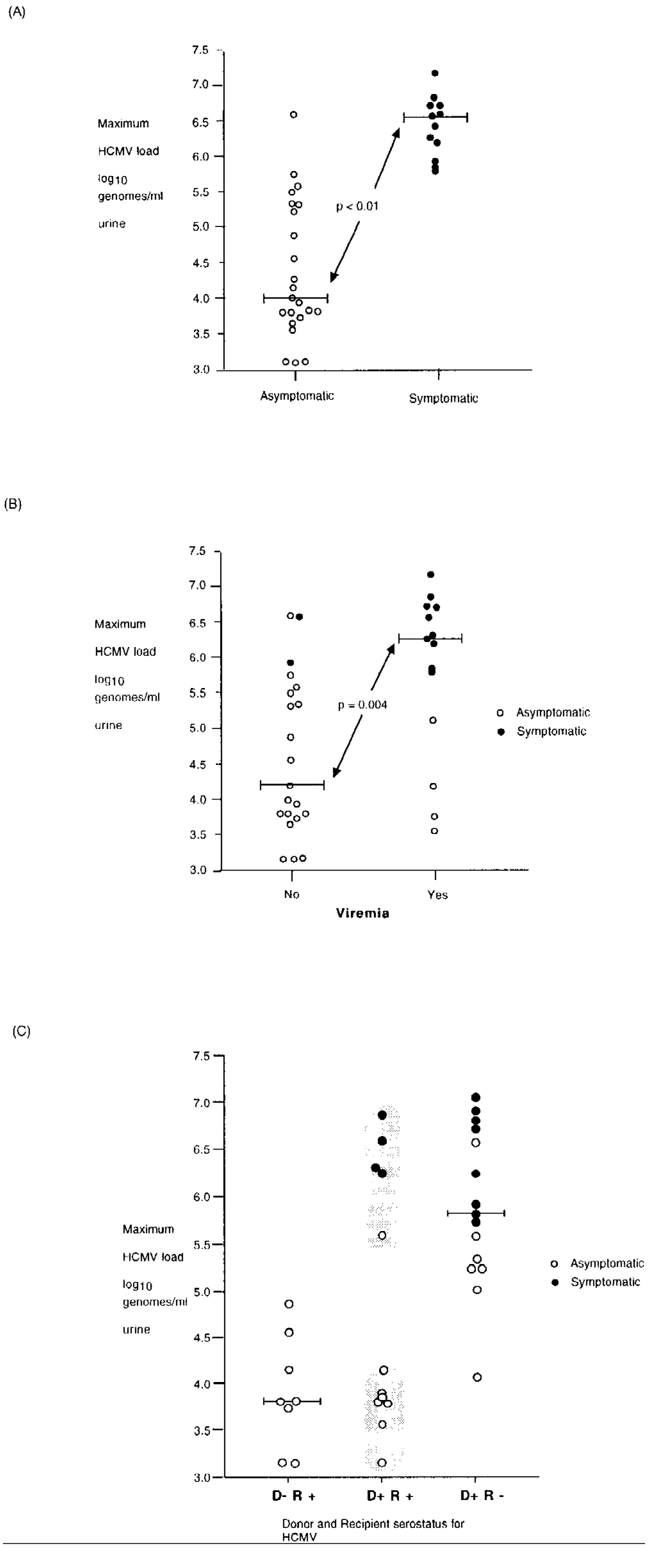

Fig. 1. Relationship between maximal viral load in the urine and HCMV disease **(A)**, viraemia **(B)**, and donor/recipient serostatus **(C)**. Maximum virus load is quoted as $\log_{10}$ genomes/ml of urine on a log scale truncated at 10^3 for clarity. ○ represents patients who remained asymptomatic. ● represents patients who experienced HCMV disease (symptomatic). The difference between the median loads in the D+R− group and D−R+ group was significant by the Mann-Whitney test ($P < 0.01$). The difference in the median viral loads between asymptomatic and symptomatic patients and between patients with or without viraemia is shown in the appropriate panel.

DISCUSSION

The results of this study clearly demonstrate that the quantity of viruria, rather than its mere presence after a renal transplant, is a significant risk factor for HCMV disease. Thus the median peak viral load in the urine post-transplant was elevated in patients experiencing symptomatic infection, those with primary HCMV infection, and those with haematogenous spread of virus. Elevated viral load, viraemia, and receipt of an organ from a seropositive donor were associated with disease in the 35 patients subjected to detailed quantitative HCMV measurements. Thus the patients in our study are representative of those previously reported in the literature because they have similar risk factors for disease [Betts et al., 1977; Goodrich et al., 1991; Irragorri et al., 1993; Ranjan et al., 1991]. Since donor kidneys are in short supply and over 50% of donors are HCMV seropositive, it would be impracticable to reject infected donors, as is done for hepatitis B, human immunodeficiency virus, and hepatitis C. Thus transplantation of HCMV-positive organs into HCMV seronegative recipients continues to occur, and clearly these patients are at highest risk of both HCMV infection and disease.

In order to analyse the relative contributions of viraemia and elevated viral load to HCMV disease, we carried out multivariate logistic regression analysis. In this analysis viral load remained a significant risk factor for HCMV disease—i.e., it was influenced only marginally by viraemia, whereas the significance of viraemia as a risk factor was substantially reduced after controlling for viral load in urine. The protective effect of recipient seropositivity against disease observed in univariate logistic regression analysis was negated once either viral load or viraemia had been controlled for. In contrast, recipient seropositivity had no marked effect on odds ratios for either viral load or viraemia in the multivariate analysis. Although the data show the dominant role of elevated viral load independent of viraemia, they do not exclude the possibility of an associated effect of viraemia. The relationship between both the probability of having disease and increasing viral load alone and with viraemia indicates that small increases in viral load above a critical threshold value (approximately $10^{5.5}$ genomes/ml urine) have a profound effect on the likelihood of developing symptoms. These findings have implications for understanding the pathogenesis of HCMV disease, in the early identification of patients at risk of HCMV disease, and in the design of strategies aimed at reducing the incidence and severity of HCMV disease.

The dominant influence of viral load determined in this longitudinal study can be used to provide insight into the pathogenesis of HCMV. For example, the observation that the temporal patterns of disease and elevated viral loads were coincident provides an explanation for the results of cross-sectional studies showing that HCMV load is higher in patients with disease [Kuhn et al., 1994; Rasmussen et al., 1995; Saltzman et al., 1992]. The observation that 2 viral load populations exist within the D+R+ group helps explain the inter-

TABLE II. Univariate and Multivariate Analysis Relating the Odds Ratio for Disease With Viral Load, Viraemia, and Recipient Serostatus for HCMV. Due to the Absence of Patients With Disease in the D–R+ Group, the Odds Ratio Associated With a Seropositive Donor was Infinity, So It Was Not Included in the Regression Analysis

Parameter	Univariate analysis Odds ratio	95% C.I.[a]	*P*-value	Multivariate analysis Odds ratio	95% C.I.[a]	*P*-value
Viral load (per 0.25 log increase)	2.79	1.22–6.39	0.02	2.77	1.07–7.18	0.04
Viraemia	23.75	3.69–153	0.0009	34.54	0.75–1599	0.07
Recipient seropositivity	0.22	0.05–0.95	0.05	0.92	0.002–446	0.98

[a]Confidence interval.

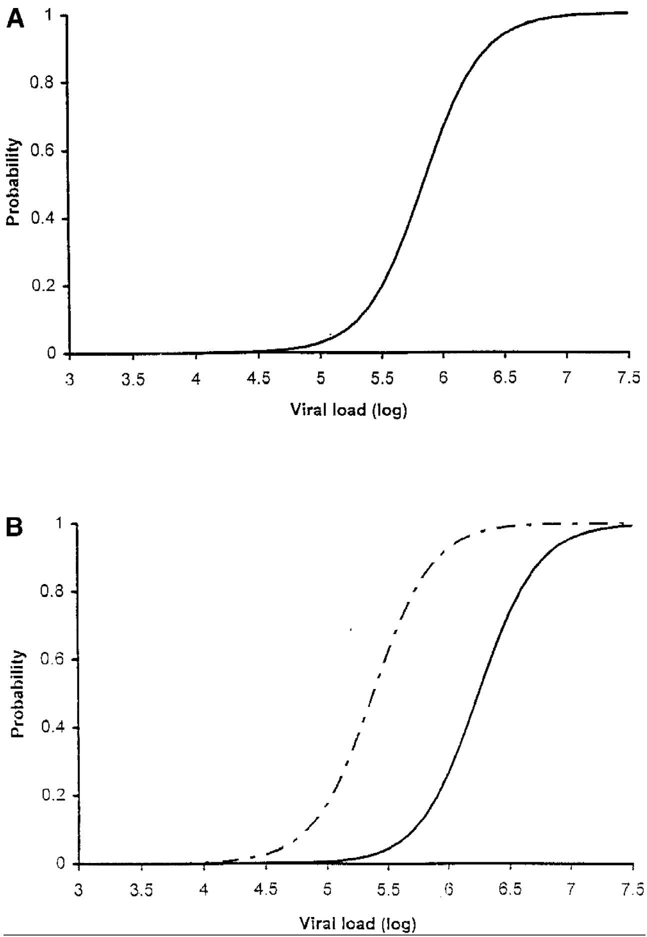

Fig. 2. **A.** Probability of HCMV disease with increasing viral load estimated from the univariate logistic regression analysis. **B.** Probability of HCMV disease with increasing viral load for viraemic (dotted line) and non-viraemic (solid line) patients, estimated from multivariate logistic regression analysis.

mediate value for the risk of HCMV disease previously observed in this donor/recipient patient subgroup. Thus individuals with low virus load (comparable to that of D–R+ individuals) are likely to be experiencing reactivation of their own HCMV and so have a low probability of disease, while the other group comprises those with an increased level of HCMV load, presumably reflecting reinfection with donor HCMV strains, which is associated with a higher probability of disease. Although this suggestion requires further support from restriction fragment length polymorphism studies, it potentially allows the identification of patients experiencing reinfection without requiring the donor strains of virus.

In order to reduce HCMV disease after renal transplant, various strategies involving the use of antiviral drugs and/or vaccines have been considered, including prophylaxis with α-interferon [Cheeseman et al., 1979; Hirsch et al., 1983; Lui et al., 1992], acyclovir [Balfour et al., 1989], immunoglobulin [Metselaar et al., 1989], or vaccination with a live, attenuated strain of HCMV [Plotkin et al., 1991]. In other transplant patient groups, suppression [Schmidt et al., 1991] or preemptive therapy [Goodrich et al., 1991] (respectively, treatment of localised or disseminated infection, detected by routine surveillance) with ganciclovir have also been used. We suggest that these and other future strategies aimed at controlling HCMV disease in renal transplant patients should be directed toward maintaining virus loads at low levels. Furthermore, the use of predetermined viral load thresholds as a proximal marker of disease could potentially provide comparative data on the relative efficacy of existing and new antiviral interventions. For example, immunisation schedules with prototype vaccines or prophylactic schedules of safe antiviral drugs could be optimised to keep virus loads below a defined threshold, without necessarily aiming to eradicate HCMV infection completely. Clearly, the objective of reducing the level of virus replication should be easier to attain than complete elimination of all replication. Drugs with potent anti-HCMV activity could then be reserved for those with high viral loads or those predicted to attain high loads, thus minimising the number of patients exposed to their toxic effects. On the basis of the results presented here, a relatively modest reduction in viral load within the range of $10^{5.5}$–10^{6} could have a significant effect on disease. In addition, threshold viral load values could be used as laboratory markers to identify patients with a high risk of future disease, and the ability to use urine for this prupose has obvious practical advantages for routine monitoring. By extension, the same principles could be applied to other patients at risk of HCMV disease, such as those receiving transplants other than kidneys, and HIV-positive patients, and we are currently investigating such patients to define the appropriate viral load thresholds.

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